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TECHNICAL REVIEW

Advancing ecological understandings through technological transformations in noninvasive genetics

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Abstract

Noninvasive genetic approaches continue to improve studies in molecular ecology, conservation genetics and related disciplines such as forensics and epidemiology. Noninvasive sampling allows genetic studies without disturbing or even seeing the target individuals. Although noninvasive genetic sampling has been used for wildlife studies since the 1990s, technological advances continue to make noninvasive approaches among the most used and rapidly advancing areas in genetics. Here, we review recent advances in noninvasive genetics and how they allow us to address important research and management questions thanks to improved techniques for DNA extraction, preservation, amplification and data analysis. We show that many advances come from the fields of forensics, human health and domestic animal health science, and suggest that molecular ecologists explore literature from these fields. Finally, we discuss how the combination of advances in each step of a noninvasive genetics study, along with fruitful areas for future research, will continually increase the power and role of noninvasive genetics in molecular ecology and conservation genetics.

Keywords: conservation genetics, forensics, genomics, molecular ecology, population genetics

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Introduction

Noninvasive genetic sampling was first used in wild animals nearly two decades ago (Höss *et al*. 1992; Taberlet & Bouvet 1992). The main advantage of noninvasive genetics is that it allows biologists to study many individuals and populations without contacting, disturbing, or even seeing the organisms. Samples collected noninvasively include faeces, hairs, urine, saliva from chewed material, feathers, scent marks, eggshells, sloughed skin, and even menstrual fluid (Table 1). The largest contributions of noninvasive approaches are to studies that focus on (i) identification of individuals for studies of population size and individual movement, (ii) wildlife forensic cases, (iii) delineation of populations and population genetic parameters (structure, gene flow and demographic history such as bottleneck detection), and (iv) assessment of mating systems and behavioural ecology (Table 1).

A growing number of noninvasive techniques yield good enough DNA and low enough genotyping error rates to allow researchers to address nearly all questions that can be addressed using traditional high-quality samples such as blood (e.g. Epps *et al*. 2006; Luikart *et al*. 2008a). This is exciting because noninvasive studies 5–10 years ago were generally more limited in scope by high genotyping error rates and low polymerase chain reaction (PCR) amplification success (reviewed in Taberlet *et al*. 1999; Waits & Paetkau 2005). In this review, we report recent advances from different research fields, hoping to open communication channels and diffuse information among disciplines.

Rapid advancements in forensic science, human medical research, and livestock disease studies, and ancient DNA techniques continuously generate

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Table 1 A list of different biological samples, taxa and purposes for which noninvasive sampling have been used in wild animal populations

	Group	Species	Purpose	Study
Blood in snow	Mammals	Wolf (*Canis lupus*)	Species identification	Scandura (2005)
Buccal and cloacal swab	Amphibians and reptiles	Tuatara (*Sphenodon punctatus*)	Methodology	Miller (2006)
Buccal swab	Amphibians and reptiles	Alpine newt (*Triturus alpestris*) and green tree frog (*Hyla arborea*)	Methodology	Broquet *et al.* (2007)a
Buccal swab	Birds	Black-capped chickadee (*Poecile atricapillus*) and boreal chickadee (*Poecile hudsonica*)	Methodology, individual and gender identification	Handel *et al.* (2006)
Eggshell	Birds	Greater snow goose (Chen caerulescens atlantica)	Methodology	Lecomte *et al.* (2006)
Eggshell	Birds	Domestic chicken (*Gallus gallus*)	Chicken anaemia virus detection	Miller *et al.* (2003)
Eggshell, feathers, buccal swab	Birds	Sage-Grouse (*Centrocercus uropihasianus*)	Gender determination	Bush *et al.* (2005)
Eggshell	Birds	Caspian tern (*Sterna caspia*) and herring gull (*Larus argentatus*)	Methodology	Schmaltz *et al.* (2006)
Faeces	Birds	Chick-rearing macaroni penguin (*Eudyptes chrysolophus*)	Diet determination	Deagle *et al.* (2007)
Faeces	Birds	European stonechat (*Saxicola torquata rubicola*)	Hormones monitoring	Goymann (2005)
Faeces	Birds	Capercaillie (*Tetrao urogallus*)	Population structure, gene flow	Regnaut *et al.* (2006)
Faeces	Mammals	Red wolf (*Canis rufus*)	Hybridization monitoring	Adams & Waits (2007)
Faeces	Mammals	Dhole (*Cuon alpinus*)	Population genetics and phylogeography	Iyengar *et al.* (2005)
Faeces	Mammals	Brown bear (*Ursus arctos*)	Population size estimation	Bellemain *et al.* (2005)
Faeces	Mammals	Wild western gorilla (*Gorilla gorilla gorilla*)	Kinship associations	Bradley *et al.* (2007)
Faeces	Mammals	Multiple fur seal species (*Arctocephalus sp*)	Diet determination	Casper *et al.* (2007)
Faeces	Mammals	Wolf (*Canis lupus*)	Population density	Creel *et al.* (2003)
Faeces	Mammals	Desert bighorn sheep (*Ovis canadensis nelsoni*)	Gene flow estimation	Epps *et al.* (2006)
Faeces	Mammals	Multiple carnivore species	Diet determination	Farrell *et al.* (2000)
Faeces	Mammals	Eurasian badger (*Meles meles*)	Population size estimation	Frantz *et al.* (2003)
Faeces	Mammals	Atlantic spotted dolphins (*Stenella frontalis*)	Methodology	Green *et al.* (2007)
Faeces	Mammals	Amur tiger (*Panthera tigris altaica*)	Individual identification by scent-marking dogs	Kerley & Salkina (2007)
Faeces	Mammals	Coyote (*Canis latrans*)	Population size estimation	Kohn *et al.* (1999)
Faeces	Mammals	Otter (*Lutra lutra*)	Methodology	Lampa *et al.* (2008)
Faeces	Mammals	Bighorn sheep (*Ovis canadensis canadensis*)	Host genetic diversity and parasitism	Luikart *et al.* (2008)a
Faeces	Mammals	Chimpanzee (*Pan troglodytes verus*)	Methodology	Morin *et al.* (2001)
Faeces	Mammals	Iberian lynx (*Lynx pardinus*)	Species identification	Palomares *et al.* (2002)
Faeces	Mammals	Rock wallaby (*Petrogale penicillata*)	Population density	Piggott *et al.* (2006)
Faeces	Mammals	Lesser horseshoe bat (*Rhinolophus hipposideros*)	Methodology	Puechmaille *et al.* (2007)
Faeces	Mammals	Kit Fox (*Vulpes macrotis mutica*)	Population genetics	Smith *et al.* (2005)
Faeces	Mammals	Western gorilla (*Gorilla gorilla gorilla*) and barbary macaque (*Macaca sylvanus*)	Methodology	Vallet *et al.* (2008)
Faeces and hair	Mammals	European pine marten (*Martes martes*) and stone marten (*Martes foina*)	Species identification	Ruiz-González *et al.* (2008)

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Table 1 (Continued)

	Group	Species	Purpose	Study
Faeces and hair	Mammals	Black bear (Ursus americanus)	Spatial behaviour	Schwartz *et al.* (2006)
Faeces and hair	Mammals	Wolverine (*Gulo gulo*)	Species and individual identification	Ulizio *et al.* (2006)
Faeces and urine	Mammals	Wolverine (*Gulo gulo*)	Individual identification and gender determination	Hedmark *et al.* (2004)
Faeces, sloughed skin and eggshell	Amphibians and reptiles	Common European viper (*Vipera berus*), ringed snake (*Natrix natrix*) and smooth snake (*Coronella austriaca*)	Methodology	Jones *et al.* (2008)
Feathers	Birds	Greater flamingo (*Phoenicopterus roseus*)	Gender determination	Balkiz *et al.* (2007)
Feathers	Birds	Powerful owl (*Ninox strenua*)	Methodology	Hogan *et al.* (2008)
Feathers	Birds	Lesser spotted eagle (*Aquila pomarina*)	Social organization	Meyburg *et al.* (2007)
Feathers	Birds	Eastern imperial eagle (*Aquila heliaca*)	Species identification	Rudnick *et al.* (2007)
Feathers	Birds	Eastern imperial eagle (*Aquila heliaca*)	Population genetics	Rudnick *et al.* (2008)
Feathers	Birds	Capercaillie (*Tetrao urogallus*)	Methodology	Segelbacher (2002)
Feathers (including museum specimens)	Birds	Spanish imperial eagle (*Aquila adalberti*)	Methodology	Horvath *et al.* (2005)
Feathers and eggshell	Birds	47 bird species	Gender determination	Jensen *et al.* (2003)
Foot mucus	Invertebrates	Multiple terrestrial snails	Methodology	Palmer *et al.* (2008)
Foot mucus	Invertebrates	Multiple intertidal snails	Methodology	Kawai *et al.* (2004)
Fresh water	Amphibians and reptiles	American bullfrog (*Rana catesbeiana*)	Species identification	Ficetola *et al.* (2008)
Hair	Mammals	Domestic dog (*Canis familiaris*)	Methodology	Bjornerfeldt & Vilà (2007)
Hair	Mammals	San Joaquin kit fox (*Vulpes macrotis mutica*)	Methodology	Bremner-Harrison *et al.* (2006)
Hair	Mammals	Black bear (*Ursus americanus*)	Population density	Dreher *et al.* (2007)
Hair	Mammals	Giant panda (*Ailuropoda melanoleuca*)	Gender determination	Durnin *et al.* (2007)
Hair	Mammals	Orang-utan (*Pongo* spp)	Methodology	Goossens *et al.* (2004)
Hair	Mammals	Multiple North American carnivores	Population genetics	Kendall & McKelvey (2008)
Hair	Mammals	Mountain pygmy-possum (*Burramys parvus*)	Genetic diversity	Mitrovski *et al.* (2007)
Hair	Mammals	Brown bear (*Ursus arctos*)	Individuals abundance	Mowat & Paetkau (2002)
Hair	Mammals	Eurasian lynx (*Lynx lynx*)	Population monitoring	Schmidt & Kowalczyk (2006)
Hair	Mammals	Southern hairy-nosed wombat (*Lasiorhinus latifrons*)	Spatial distribution and habitat use	Walker *et al.* (2008)
Hair	Mammals	Ocelot (*Leopardus pardalis*)	Species, gender individual identification	Weaver *et al.* (2005)
Hair	Mammals	Multiple carnivore species	Methodology	Zielinski *et al.* (2006)
Hair/faeces/ urine/tooth/saliva	Mammals	Wolf (*Canis lupus*)	Gender determination	Sastre *et al.* (2008)
Insect exuviae/frass	Invertebrates	Multiple butterfly species	Species identification	Feinstein (2004)
Ivory	Mammals	African Elephant (*Loxodonta africana spp*)	Forensic cases	Wasser *et al.* (2007)
Menstrual bleeding	Mammals	Taiwan macaque (*Macaca cyclopis*)	Methodology	Chu *et al.* (1999)
Museum specimen	Birds	*Gallinago spp*	Methodology	Lee & Prys-Jones (2008)

Table 1 (Continued)

	Group	Species	Purpose	Study
Museum specimen	Mammals	Brown bear (*Ursus arctos*)	Phylogeography	Leonard *et al.* (2000)
Museum specimen	Mammals	Stoat (*Mustela erminea*)	Methodology	Martinkova & Searle (2006)
Museum specimen	Mammals	Wolverine (*Gulo gulo*)	Evolutionary significant units	Schwartz *et al.* (2007)
Regurgitate	Mammals	Wolf (*Canis lupus*)	Individuals dispersion	Valière & Taberlet (2000)
Saliva	Birds	Common marmoset (*Callithrix jacchus*)	Cortisol levels and behavioural stress	Cross *et al.* (2004)
Saliva	Mammals	Wild chimpanzee (*Pan troglodytes verus*)	Individual identification	Inoue *et al.* (2007)
Saliva	Mammals	Wolf (*Canis lupus*)	Predator identification	Sundqvist *et al.* (2008)
Saliva	Mammals	Coyote (*Canis latrans*)	Predator identification	Blejwas *et al.* (2006)
Scent mark	Mammals	Multiple murine species	Microbial parasite communities identification	Lanyon *et al.* (2007)
Skin, blubber and meat	Mammals	Pacific minke whale (*Balaenoptera acutorostrata* spp)	Forensic cases	Baker *et al.* (2007)
Sloughed/shed skin	Mammals	Humpback whale (*Megaptera novaeangliae*)	Methodology	Elphinstone *et al.* (2003)
Sloughed/shed skin	Mammals	Humpback whale (*Megaptera novaeangliae*)	Individuals abundance	Palsboll *et al.* (1997)
Sloughed/shed skin	Mammals	Ringed seal (*Phoca hispida*)	Methodology	Swanson *et al.* (2006)
Urine	Mammals	Japanese macaques (*Macaca fuscata*)	Methodology	Hayakawa & Takenaka (1999)
Urine	Mammals	Wolverine (*Gulo gulo*)	Methodology	Hedmark *et al.* (2004)
Urine	Mammals	Wolf (*Canis lupus*)	Population monitoring	Hausknecht *et al.* (2007)
Urine	Mammals	Multiple canid species	Species and individual identification	Valière & Taberlet (2000)
Urine	Mammals	Wolf (*Canis lupus*)	Molecular sexing	Sastre *et al.* (2008)

improved techniques that can be applied in noninvasive genetics to improve both data production and analysis. Unfortunately, these scientific communities seldom cross-reference each other. To continually improve molecular ecology and conservation genetic studies, we recommend that researchers occasionally search for novel approaches in journals from diverse fields including forensics (e.g. *Journal of Forensic Sciences*), human and animal health (*Avian Disease*, *New England Journal of Medicine*), microbiology (e.g. *Journal of Applied Microbiology*), biochemistry and biotechniques (*Analytical Biochemistry* and *Nature Methods*), and bioinformatics, e.g. *Biometrika* (see also our literature cited).

This review is structured around the steps in a noninvasive study, from pre-PCR sampling to post-PCR data analysis, and concludes with perspectives for future research. Noninvasive studies should not be seen as a one-step process, but as a chain of steps that should be monitored independently. The chain starts in the living animal and ends only when the statistical analyses of the final data provide convincing evidence that results and conclusions are reliable. We consider five major steps to be monitored and how to avoid pitfalls and improve noninvasive studies (Fig. 1). Accordingly, this review is structured around steps and techniques, not research questions (e.g. paternity analysis, population structure), which allows readers to quickly go to the step or technique of interest (pre-PCR to post-PCR) to find information.

Pre-PCR

Obtaining samples

Creative ways to noninvasively obtain DNA from numerous types of samples are continuously being developed, improved and evaluated (Table 1). The collection of everything from menstrual fluid to mucus trails left by snails has been used to identify species and individuals noninvasively (Table 1). Several sample types can be obtained by following a trail of an animal on natural surfaces such as snow or sand, without ever seeing the target animals. For example, Ulizio *et al.* (2006) collected 169 hair samples and 58 scat samples on 54 wolverine backtracks.

One creative study reported the noninvasive detection of species (a frog, *Rana catesbeiana*) in natural wetlands by PCR testing for mtDNA in water samples (Ficetola *et al.* 2008).

Faeces are one of the most commonly used noninvasive materials because, for many species, it is the easiest to find in the wild and it provides more information (e.g. diet, stress hormone status, reproductive hormones, parasite infection and parasite DNA) than other sample

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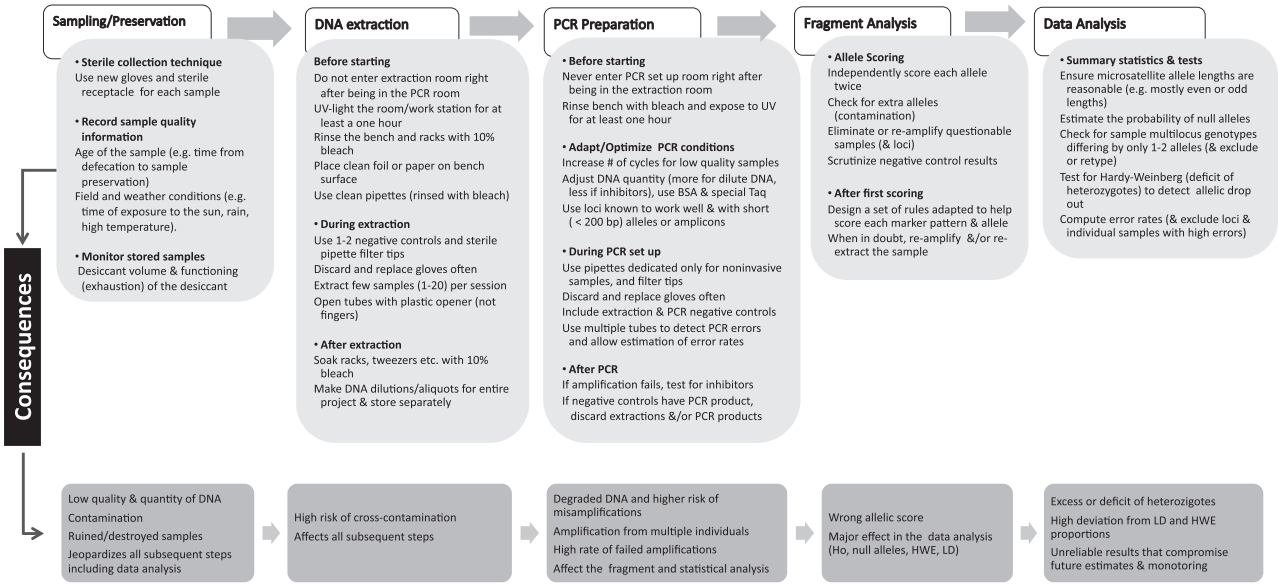

Fig. 1 Schematic representation of some critical points (light grey lists) that should be checked at each of the five main steps (white rectangles on top) of the noninvasive samples processing. Below (arrow boxes) are some likely consequences of not correctly following and monitoring these points. Some points are common sense and widely known but nonetheless are often violated. HWE, Hardy–Weinberg equilibrium proportions; LD, linkage- or genotypic-disequilibrium.

types (Kohn & Wayne 1997; Goymann 2005; Luikart *et al.* 2008a; Schwartz & Monfort 2008). Faeces in some species (e.g. ungulates, arboreal primates, macropods, etc.) can be collected just after observing individuals defaecate without disturbing the animals. An enormous advantage of observing the target animals is that the faeces are fresh and DNA is relatively little degraded. It can also help avoid collecting from nontarget species and determine sex (by observation) and thus avoid need for DNA-based species identification and sexing (Epps *et al.* 2006; Luikart *et al.* 2008b). In secretive or elusive species such as forest ungulates, bears, fishers, mountain lions and tigers, domestic dogs can be trained to find faeces (reviewed in McKay *et al.* 2008). Dogs can also identify individual animals, as was shown in a study of faeces from known tigers (Kerley & Salkina 2007). Hair is another widely collected material (Table 1). In apes (e.g. orangutans, chimpanzees), individuals build a new nest every night and hair that is shed during the night can be found in the nests. Researchers recommend using only hairs with visible root bulbs as many shed hairs do not contain large bulbs with DNA (Goossens *et al.* 2004). In a study of wolves, hair (along with faeces, urine and saliva) allowed highly successful DNA amplification (93% of samples) for noninvasive sexing of individuals using sex chromosome markers (Sastre *et al.* 2008). Hair is also often recovered frozen in the snow tracks of felids and canids and in bed sites of ungulates.

Many hair snare devices have been invented (e.g. Bremner-Harrison *et al.* 2006; Zielinski *et al.* 2006) for noninvasive sampling. Hair snares are used to sample bears (e.g. Immell & Anthony 2008; Kendall *et al.* 2009), felids (e.g. Weaver *et al.* 2005; Schmidt & Kowalczyk 2006) and mustelids Mowat & Paetkau 2002. Barbed wire or sticky tape is also often strung around bait stations or draped across animal burrow entrances to pluck hairs when animals pass by (Pauli *et al.* 2008; Tóth 2008; Walker *et al.* 2008). Along with hair snaring devices, commercial lures (such as catnip and valerian oils, among other attractants) have been successfully used to attract and elicit cheek-rubbing behaviour in felid species (e.g. *Lynx canadensis*, McDaniel *et al.* 2000). For hair snares, a potential advantage is that they obtain plucked hairs, which generally contain more and larger root bulbs (with cells and DNA) than shed hairs. However, it might be difficult avoiding cross-contamination between individuals because multiple individuals can be sampled before hairs are recovered from the snare. As birds use mammal hair to strengthen the structure of their nest, recently Tóth (2008) used bird nests as sources of hair samples and identify mammals that occupy or migrate through a specific area.

Feathers have repeatedly been shown to be a good source of DNA. Shed feathers can be collected from nests. Feather snares (e.g. sticky tape) potentially could help obtain feather samples, but to our knowledge have not been reported in the literature. A particularly informative and recent study (Hogan *et al.* 2008) showed that different feather types (down, semi plume, contour or remige/rectrice) yield useful DNA. However, feather

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condition (as estimated from physical appearance) strongly influenced PCR amplification success.

For eggshells, a recent study used cotton swabs to obtain DNA from the external shells of herring gull (*Larus argentatus*) and Caspian tern (*Sterna caspia*) eggs (Handel *et al.* 2006). Researchers verified that the DNA samples were maternal (not the chick's) by comparing microsatellite profiles with those obtained from adults and chicks from the same nests. In all of 28 tests, the egg swabs matched the maternal microsatellite genotype. In a screening of many nests of both species, microsatellite markers were successfully amplified from egg swabs. Eggshells are also used as a source of DNA in veterinary health and disease studies (e.g. Miller *et al.* 2003), from which molecular ecologists might learn new and useful techniques (e.g. improved DNA extraction or PCR techniques).

Eggshells, feathers and mouth swabs from sage-grouse (*Centrocercus urophasianus*) were compared for PCR success in one of the few studies directly comparing multiple sample types (Bush *et al.* 2005). These authors found hatched eggshell membranes yield useful DNA (better than predated eggshells), as did plucked body contour feathers, chick down feathers and mouth swabs. However, allelic dropout rates of approximately 10% were observed for eggshells, and moulted feathers had only 60% PCR amplification success (Bush *et al.* 2005).

Saliva is also a good source of DNA. It is often used in forensics, for example, to recover DNA from bite marks found in homicides, assault and other criminal cases (Anzai-Kanto *et al.* 2005). In wildlife, Williams *et al.* (2003) used saliva collected from sheep bite wounds to identify the canid species responsible for attacks on domestic sheep; the authors identified the predator species (coyote) and determined the sex of the individual. Saliva is also used to solve cases of livestock attacks in which wolves and dogs are the main suspects (Sundqvist *et al.* 2008).

For some sample types, a new swab sampling technique reported in the forensics literature could improve the quality of genotyping Pang & Cheung, 2007. The double swab technique, using a wet cotton swab followed by a dry cotton swab, was compared with the classical technique (one wet swab) for recovering DNA from evidence collected at crime scenes. Swab techniques could potentially improve noninvasive sampling studies involving material such as eggshells or any surfaces that animals come into contact, rub against, lick or bite (e.g. rocks, sticks). Further evaluation of this and other sampling methods is needed.

Preserving DNA in noninvasive samples

A growing diversity of protocols exists for preserving DNA in samples. This makes it difficult for researchers to understand which protocol is most reliable, most thoroughly validated, or requires further development and testing. For noninvasive samples, it is essential to conduct a pilot study using the exact target material, preservation method and extraction technique to ensure recovery of sufficient DNA (Bhagavatula & Singh 2006; Valière *et al.* 2006; Schwartz & Monfort 2008).

The preservation of DNA in a noninvasive sample is a race to inhibit enzymes that degrade DNA, i.e. nucleases. There are three main approaches used to preserve samples: deactivation of nucleases via removal of water, deactivation of nucleases via the elimination of cations (e.g. $MgCl_2$; Thomas & Gilbert 2006) and inhibition of nuclease activity via storage of samples at low temperatures. Removal of water is achieved using drying agents (e.g. ethanol, silica gel) or drying techniques (e.g. vacuum spinning, lyophilization, oven heating). Removal of cations is achieved using chelators such as EDTA or resin (e.g. Chelex®). Insufficient volumes of preservatives (ethanol or silica) or failure to freeze samples quickly often leads to DNA degradation. Several published studies comparing different preservation protocols can help researchers choose the best protocol according to their samples (Roon *et al.* 2003; Hajkova *et al.* 2006; Broquet *et al.* 2007b; Santini *et al.* 2007). Nonetheless, there are inconsistencies among some studies, and even some suggestion that there is an interaction between preservation techniques and extraction methods (Piggott & Taylor 2003).

A potentially improved preservation approach is to combine use of silica and ethanol (ETOH) protocols, however, the ETOH (90%) performed similar to the combined two-step method when using lower quality samples (Roeder *et al.* 2004). This combined approach was repeated on gorilla and chimpanzee faeces and yielded more DNA than silica alone or RNAlater alone (Nsubuga *et al.* 2004). Nonetheless, this approach has not been extensively compared with other methods (e.g. ETOH 97%) in a wide variety of species. Long-term preservation might be improved by the addition of trehalose as a preservative agent (Smith & Morin 2005), although this method has not been independently evaluated. There is a great need for comparative evaluations of most preservation methods.

For faeces preservation, it is difficult to decide which desiccant (e.g. ETOH, silica, salts) should be used. A large amount of any desiccant should be used per sample (e.g. 5–10 parts of desiccant per part of sample) to rapidly and completely dry the sample material. Given the wide use and success, we recommend the use of ETOH in large volumes (5–10 times the sample volume) and in high concentration (≥95% ETOH).

With faecal material, ETOH has advantages over silica in that ETOH prevents formation of faecal powders (thus cross-contamination by aerosol). It also keeps the external

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mucous layer containing cells packed against faecal material, whereas silica can be abrasive and can remove mucus and cells from outer surface of the faeces (e.g. during transportation and shaking of samples). ETOH has a notable disadvantage in being flammable and therefore potentially dangerous and more expensive to ship via airplane. As an alternative, silica is useful and widely tested but again requires large volumes of this mineral to ensure rapid drying and to avoid exhausting the desiccation function.

For faeces, RNA later® might be a better preservative than ETOH or silica http://www.aim.uzh.ch/orangutannetwork/GeneticSamplingProtocol.html#18; Nsubuga *et al.* 2004). RNA later® is a solution meant for preserving RNA in tissue. However, the solution is expensive (US$2–4 depending on volume needed per sample), and further research is needed to formally test and compare it with other preservatives.

For hair samples, the most common storage method is simply to store it (shed or plucked) in a dry envelope often with silica gel granules at room temperature (Jeffery *et al.* 2007). A fairly thorough comparative study of freezing (−20 °C) vs. silica desiccant found that freezing gives slightly higher (though nonsignificant) amplification success for both microsatellites and mtDNA from brown bear hair, *Ursus arctos* (Roon *et al.* 2003). Amplification success was above approximately 90% up to 6 months of storage but dropped below approximately 80% between 6 and 12 months for both the 1000-bp mtDNA fragment and three microsatellite loci. More comparative studies are needed using different preservation methods including a *combination* of freezing and silica gel, and perhaps storing hairs immediately into a lysis or storage buffer solution. Sorting hairs based on root bulb size and quality should also be conducted to maximize amplification success and perhaps improve accuracy of comparisons among preservation methods (Jeffery *et al.* 2007).

For feathers, storage in paper envelopes at −20° allowed successful amplification of mtDNA and nDNA from powerful owls (*Ninox strenua;* Hogan *et al.* 2008). In this study, the paper envelopes containing 637 shed feathers were stored in plastic bags in dry and dark conditions for up to 7 months. Amplification success was 80–90% for mtDNA and microsatellites on feathers in good condition but only 30–40% for feathers in poor condition (with visible physical degradation of calamus and barbs on the vane). Feather type had no effect on amplification success. We recommend against using plastic bags as humidity can potentially build up inside, unless silica desiccant is inside the bag. Feathers from adult eagles (Rudnick *et al.* 2007) stored dry at room temperature yielded microsatellite genotypes using a pre-amplification PCR method (PCR section below), although nearly 10% of samples yielded no PCR product. In the same study, developing chick feathers were stored at room temperature in a lyses buffer (EDTA, SDS) for several months before being ultimately stored at −80 °C up to several years before yielding microsatellite genotypes.

Saliva samples are generally preserved by freezing at −20 °C (Anzai-Kanto *et al.* 2005). For example, Anzai-Kanto *et al.* (2005) published a study using human saliva in which they estimate that 0.3 mL of saliva is enough to provide DNA for genotyping 15 loci. Swabs are the most general method to sample buccal/oral DNA, and these swabs are generally dried at room temperature followed by freezing at −20 °C or even colder temperatures (e.g. see Sundqvist *et al.* 2008).

Urine samples as a source of DNA have been increasingly used in recent years. Urine can be collected using a swab to swipe the surface location where the animal urinated (e.g. rocks, sticks, leaves). The swab will absorb the urine together with the cells. Another method, used in winter, is the collection of urine in snow (yellow snow). Researchers have melted yellow snow in a 15-mL tube, which will contain urine, cells and DNA (Hausknecht *et al.* 2007). This method has been tested in carnivores, in particular the wolf. Urine samples can also be collected from soil samples. We have collected fresh ungulate urine from dirt, which becomes mud (G. Luikart, unpublished). We stored the urine mud in six volumes of 95% ETOH, similar to faecal samples, until extraction in the laboratory using stool extraction kits or soil kits (see below). While urine can be a useful material, it often has a lower amplification success rate as compared with other noninvasive samples (Hedmark *et al.* 2004). Hedmark *et al.* noticed a decline in microsatellite amplification success of wolverine urine (40% success) as compared with faeces (65% success).

Extracting DNA from noninvasive samples

DNA extraction is a crucial step, because all subsequent steps in a genetic project hinge upon extraction quality. Phenol/chloroform extraction methods were the most widely used 10–15 years ago, but now are seldom used, mostly because the chemicals are hazardous, the approach is time-consuming, and sometimes PCR inhibitors remain after extraction. As alternatives, different methods have appeared, most of them imported from forensic genetics (e.g. see book by Morling 2008).

Resin-based (e.g. Chelex®) extractions are widely used for noninvasively collected samples. Chelex is useful for extracting DNA from hair follicles (Mitrovski *et al.* 2005; Koukoulas *et al.* 2008), stains at crime scenes, and even for formalin-fixed archived tissues (Chakraborty *et al.* 2006). Its main advantages are speed and low cost (http://bugs.bio.usyd.edu.au/DNA/DNAextrn.html).

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The main disadvantages are that (i) DNA extracts are not always highly pure, (ii) DNA can degrade after several months, and (iii) Chelex itself can inhibit the PCR amplification (Willard *et al.* 1998).

Commercial kits for extracting DNA are also widely used. Among these, the most common are silica-based spin column kits. The working principle of this method involves the lysis of the cell membranes (e.g. by detergents and proteinase K), followed by purification using silica-based compounds in spin columns that bind and then allow washing of DNA (Boom *et al.* 1990). The great success of these kits results from their ease of use and adaptability to a wide range of biological samples (e.g. plant tissues, bacteria growing media, skin, muscle, bone, faeces, urine, blood, museum skins, ancient bone) with minimum changes.

When comparing five DNA extraction methods, the extracted samples from which a fragment of 149 bp of the mtDNA was successfully PCR amplified using a commercial kit (QIAGEN Stool DNA extraction kit) was 100%, followed by 88% using guanidinium thiocyanate-silica, 75% for the digest buffer/phenol–chloroform, 38% for chelex-100 and 25% for the lyses buffer/column purification method (Bhagavatula & Singh 2006)

For pellet-form faeces, which are amenable to a surface wash, the wash technique combined with commercial extraction kits [e.g. DNeasy^{TM} Blood Kit (QIAGEN)] has been highly successful. The washing step is a simple 10–15 min incubation of a faecal pellet in a buffer solution followed by extraction of DNA from the buffer using a blood DNA extraction kit (Luikart *et al.* 2008b). The surface-wash liquid contains relatively few PCR inhibitors and therefore does not always require use of the more expensive and time-consuming 'stool kits' with additional steps to remove inhibitors. This approach yields high amplification success, low genotyping error rates and large quantities of DNA.

For faeces, a cell enrichment method has been reported to recover large quantities of high molecular weight DNA (Wan *et al.* 2006). The cell enrichment based protocol is so far the only one that deals with large quantity of faeces, and is based on the soaking in a large volume of buffer to disperse the faecal material completely. A commercial company (Noninvasive Technologies) offers a kit for a similar extraction, but it costs over US$200 for the extraction of two individual samples. With faecal (& urine) samples, it is difficult to quantify the amount of extracted DNA using conventional methods (e.g. spectrophotometer) because these are inefficient with trace quantities of DNA, they cannot estimate DNA degradation, nor can they differentiate between DNA from the target species or microbes often in faecal (& urine) DNA extractions. To cope with these limitations, several assays have been developed using real-time quantitative (RTQ) PCR (Morin *et al.* 2007). Unfortunately, RTQ-PCR still is not affordable for all laboratories and alternative low-cost methods can be used to quantify the DNA extracted from some noninvasive samples. For example, Ball *et al.* (2007) used a method based on Picogreen^{TM} (Molecular probes), a fluorescent dye, to measure the amount of double-stranded DNA extracted from noninvasive samples (e.g. faeces). Picogreen^{TM} binds double-stranded DNA and when excited by laser releases a florescent signal that is proportional to the amount of double-stranded DNA present in the tested aliquot. However, unlike RTQ-PCR, fluorescent dye methods cannot differentiate between the target species vs. microbial DNA.

The urine samples can be collected either by using a swab across the surface where the animal urinated (e.g. rocks, bush leafs) or in winter from snow. One extraction method involves centrifuging cells (sloughed off from the epithelium of the urinary tract). Once the cells are collected in a pellet, standard DNA extraction protocols can be used (Hausknecht *et al.* 2007). This approach is also valid for buccal-mouth wash (nondestructive) sampling in humans (Mayntz-Press & Ballantyne 2007). Another extraction method directly precipitates DNA from the sample (e.g. snow) containing the urine (Valière & Taberlet 2000). Direct precipitation would be advantageous when cells burst and DNA is free. DNA from the urine deposited in the soil (mud) can be obtained using stool DNA extraction kits or soil DNA extraction kits (e.g. Thakuria *et al.* 2009). Comparative evaluations of extraction kits on humans suggest that some kits (miniMAG) yield far better DNA than others, including DNA from pathogens being monitored noninvasively (Tang *et al.* 2005). Noninvasive wildlife studies might benefit from testing and using kits used in human studies.

For hairs, an improved extraction method reported use of Ca^{+} to increase digestion and release of DNA of hair shafts. In a forensic-based study of hairs from 170 dogs from different breeds, the quantity of DNA extracted increased 100% when compared to the well-established QIAGEN tissue kit (Pfeiffer *et al.* 2004).

Finally, it is important to mention that plastic tubes may have a strong effect of reducing DNA quantities when the amount of DNA in the sample is very low (fewer than 1000 target copies) because of DNA adhering to the plastic walls of the tube. A recent study showed that use of low-retention plastic tubes significantly reduce DNA loss, but DNA from nontarget species added to prevent the loss of target DNA had no effects (Ellison *et al.* 2006). As this problem becomes better understood, we imagine that low-retention plastic tubes will drop in price; more research is needed on changes in DNA yield caused by tube choice.

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Polymerase chain reaction

Here we review approaches to improve PCR amplification of DNA, including pre-PCR treatments (for inhibitors and broken DNA fragments), amplification of smaller fragments (mini-STRs/microsatellites and SNPs), nested PCR techniques, different *Taq* polymerase enzymes and genotype scoring criteria.

Overcoming PCR inhibitors

Inhibition of PCR can cause low amplification rates, even in samples with abundant DNA and apparently suitable for PCR (Kontanis & Reed 2006). For example, faeces contain compounds that can be strong PCR inhibitors, including complex polysaccharides, products from food degradation (e.g. acids, secondary plant compounds, enzymes, lipids and proteins), RNA and bacteria. As previously discussed, DNA extraction protocols combined with washes for DNA purification are essential to remove inhibitors. However, some inhibitors may still remain and result in amplification failure.

Dilution of the DNA extracts is the simplest way to reduce inhibitors (dilution is the solution to pollution). For example, Thornton & Passen (2004) diluted approximately 256-fold the DNA extract obtained from 10 mg of bovine faeces to achieve amplification inhibited by phytic acid (present in plants). Dilution also increased amplification efficiency of Iberian lynx (*Lynx pardinus*) mtDNA from 92.6% to 99%, equivalent to the benefit of performing a second amplification for each sample (Palomares *et al.* 2002). However, genotyping errors can be caused by low target DNA quantity or the presence of PCR inhibitors (or both interacting). Accordingly, a balance between diluting PCR inhibitors and over-diluting the DNA in the extract often must be established.

Precipitation of DNA (e.g. with ETOH) also removes inhibitors (and increase DNA concentration). This involves a washing step of the DNA pellet before re-dissolving the DNA precipitant in water or buffer. Addition of PCR adjuvants such as bovine serum albumin (BSA), dimethyl sulfoxide, or nonionic detergents (e.g. Tween 20 and Triton X-100) often binds inhibitors and improves amplification specificity. Most noninvasive studies include an additive in PCR protocols. BSA is the most widely used adjuvant (from 0.1 to 1.2 μg/μL in concentration) because it seldom interferes with PCR in the absence of an inhibitor.

Overcoming DNA degradation and fragmentation

Using very short fragments such as mini short tandem repeats (mini-STRs, also called mini-microsatellies) or single nucleotide polymorphisms (SNPs) can help overcome difficulties amplifying degraded DNA (e.g. Campbell & Narum 2008). In several noninvasive studies, long amplicons (>200–300 bp) produced significantly higher allelic dropout rates than short amplicons (Broquet & Petit 2004; Buchan *et al.* 2005). Several studies have redesigned primers to produce shorter amplicons and improve microsatellite analysis in forensic research (e.g. Butler *et al.* 2003; Chung *et al.* 2004). In fact, studies using historical or ancient DNA typically amplify multiple small (100 bp) regions, instead of one large region as is typical with high-quality DNA (Schwartz *et al.* 2007).

Single nucleotide polymorphism studies can achieve higher amplification success and lower error rates than microsatellites, because SNP amplicons are generally shorter (<100 bp) than microsatellite amplicons (100–300 bp). For example, Musgrave-Brown *et al.* (2007) showed that a 52-plex SNP assay performed better than STR (microsatellite) typing on degraded samples. However, the biallelic nature (and thus limited heterozygosity) of SNPs must be compensated by typing a larger number of SNP loci (Morin *et al.* 2004, 2009a, b). Thus, even if there is a lower error rate per SNP, the amplification of many more SNPs may cumulatively increase the overall (multilocus) genotyping error rates. More research is needed to quantify the increase in multilocus error rates when adding more loci because the increase can be unpredictable given that errors are often not randomly distributed among PCRs, alleles and loci (Pompanon *et al.* 2005).

The benefit of amplifying shorter SNP fragments is likely to outweigh the lower variation and need to include more loci when using SNPs. For example, Campbell & Narum (2008) genotyped chinook salmon samples of varying quality with 13 microsatellite and 29 SNP assays and the average genotyping success for good, intermediate and poor quality samples was 98%, 97% and 79% for SNPs but only 96%, 24% and 24% for microsatellite loci respectively. Few studies have quantified genotyping error rate using SNPs in noninvasive or historical samples. Morin & Mccarthy (2007) used 19 SNPs in a study using historical samples of bowhead whales; they found a 0.1% genotyping error rate, which is lower than most noninvasive studies.

During PCR, broken DNA fragments may anneal to each other and form priming sites needed for amplification, resulting in different sized fragments and the scoring of false alleles. To prevent this unwanted production of chimeric alleles (e.g. DNA fragments that anneal together giving the appearance of another allele) and to avoid the occurrence of jumping PCR (recombination between similar DNA sequences during PCR that is promoted in damaged/fragmented DNA), Čuljković *et al.* (2003) described a pretreatment of DNA fragments before PCR by adding a poly(A) tail at the 3′ prime end of

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templates to eliminate homology between fragments. This has been successfully used in ancient DNA studies, but not to our knowledge in noninvasive studies.

Overcoming low DNA quantity

Several PCR-based strategies to overcome problems associated with low-quantity DNA have been proposed recently. Pre-amplification (i.e. double amplification) is an efficient procedure to increase the amount of low copy number template because products from a first amplification are used as templates for a subsequent PCR; this pre-amplification increases the DNA available for the second desired amplification (e.g. Lau *et al.* 2003). A second PCR with internal (nested) primers can also increase genotyping success and specificity to amplify only the target locus because the internal primers (as well initial external primers) can be locus specific. The same is true when using only one internal primer in the second PCR (Bellemain & Taberlet 2004). A semi-nested or second PCR can be especially useful to improve amplification of certain difficult loci. A second PCR is also useful after whole genome pre-amplification or multiplex pre-amplification.

Whole genome amplification is the production of amplicons across an entire genome to increase the amount of template DNA available for subsequent locus-specific genotyping (Kittler *et al.* 2002). This approach has been successfully applied before genotyping microsatellites, although preferential amplification of the shorter alleles might occur. Similarly, whole-genome amplification with degenerate primers (i.e. mixtures of similar, but not identical, primers) has been successfully used for large-scale SNP genotyping despite a detectable loss in genotype accuracy (Grant *et al.* 2002). In some studies, as the one reported by Vigilant (1999) in genotyping shed chimpanzee hairs, this strategy was ineffective for improving microsatellite genotyping.

Pre-amplification of multiple loci in a multiplex can improve microsatellite genotyping from noninvasive samples (Box 1). This method can increase the quantity of target DNA fragments for each locus while minimizing consumption of the initial DNA extract. In this approach,

Box 1. The promise of real-time quantitative PCR

Real-time quantitative PCR quantifies the amount of target-specific, 'amplifiable' DNA from an extraction. This is important because DNA might exist in a sample (e.g. quantified by fluorometry), but not be amplifiable because of PCR inhibitors, extreme DNA fragmentation, and/or the DNA is from nontarget species. RTQ-PCR differs from regular PCR in that the PCR product is quantified as the PCR is occurring, using a fluorescent dye. In each PCR cycle, the amount of the target locus DNA doubles and so does the fluoresce intensity. An RTQ-PCR machine is a PCR machine with a fluorometer. Advantages of RTQ-PCR are its sensitivity (it is the most sensitive PCR method for low quantity of DNA) and that there is no post-PCR manipulation of samples (gel electrophoresis); this saves time and money, and avoids contamination as post-PCR tubes are never opened in the laboratory.

Real-time quantitative PCR has enormous (largely untapped) potential to improve noninvasive studies by identifying samples with enough nuclear DNA to avoid genotyping errors. The amount of DNA necessary to avoid genotyping errors (allelic dropout) has been estimated to be approximately 100–600 pg by theoretical and empirical studies (e.g. Taberlet *et al.* 1996; Morin *et al.* 2001). RTQ-PCR could improve noninvasive studies by excluding extremely low quality samples and identifying samples at risk of having genotyping errors.

A single RTQ-PCR can identify species in addition to quantifying amplifiable DNA (Berry & Sarre 2007). Species identification is possible if species-specific primers are used or if the targeted PCR product has a different melting curve (Berry & Sarre 2007). RTQ-PCR could replace species identification methods, which often involve mtDNA analysis and that currently are the standard first step in many noninvasive studies (e.g. Swango *et al.* 2006).

The first paper using RTQ-PCR on noninvasive samples was Morin *et al.* (2001). Subsequently, the same RTQ-PCR was used on ape faeces to identify factors (e.g. temperature) and sample preservation methods (ethanol and silica) that improve PCR amplification. Several recent papers report successful RTQ-PCR of DNA from faeces and urine, although most papers involve testing for cancer genes or disease pathogens in humans or livestock (e.g. Inglis & Kalischuk 2004; Queipo-Ortuño *et al.* 2006; Itzkowitz *et al.* 2007). These recent papers are highly encouraging and suggest that RTQ-PCR from faeces and urine is highly feasible and efficient.

We expect that RTQ-PCR will be widely used in future noninvasive studies because the methods have become easier (e.g. with commercial kits), less expensive, and clearly work on noninvasive samples (Hausknecht *et al.* 2007). An RTQ-PCR reaction can cost as little as approximately US$1 per PCR (e.g. Berry & Sarre 2007). The cheapest RTQ-PCR method (SYBR green) is also often highly reliable (e.g. Smith *et al.* 2002). An RTQ-PCR machine costs approximately US$15 000–30 000 and prices are likely continue to fall (e.g. see http://www.biocompare.com/matrix/2838/Real-Time-PCR-ermalCyclers(Thermocyclers).html).

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an initial large-volume PCR with all primer pairs is performed followed by a second or nested PCR of each genetic marker (Piggott *et al.* 2004). The use of this two-step PCR approach revealed significant improvements in efficiency relative to standard PCR (Piggott *et al.* 2004; Hedmark & Ellegren 2005; Arandjelovic *et al.* in press). Because it requires less DNA extract, multiplex pre-amplification allows typing more loci, which is often a limitation in noninvasive genetics.

However, multiplex pre-amplification has drawbacks. Allelic dropout can occur more frequently than for conventional PCR, suggesting that this type of error is often generated during the first-step multiplex (Lampa *et al.* 2008). In addition, a multiplex might increase the proportion of nonamplifiable loci because of the competition between loci (Lampa *et al.* 2008). Alternatively, genotypes can be obtained by performing additional single standard PCR whenever single locus amplification remains the most suitable approach to satisfy efficiency and accuracy (Parsons 2001). Although nested PCR increases the efficacy and sensitivity for amplifying target genomic fragments, it has the drawback of increasing the risk of contamination, because it requires two PCR reactions and, consequently, doubles the handling of materials. This problem might be particularly prominent for noninvasive studies.

Overcoming nonspecific amplification and contamination

Co-amplification of nonspecific products and contamination can be major problems in noninvasive genetics. PCR with low quality and quantity target DNA can increase the probability of amplifying nontarget regions. It also increases the probability that contaminant DNA is at similar or higher concentrations than target DNA (Pompanon *et al.* 2005). Navidi *et al.* (1992) estimated that sporadic contamination could cause up to 7% error in large-scale studies, and Buchan *et al.* (2005) estimated that 1.3% of the baboon DNA analysed and 1.2% of the negative controls of their study were contaminated with human DNA.

Hot start PCR is one of the most effective means to improve specificity, fidelity and sensitivity in DNA amplifications. Effective protocols are now widely available thanks to the use of engineered thermostable polymerases (whether using an inhibitor antibody or chemical modification) that require heat activation prior to PCR cycling, and because of the use of high-performance PCR buffers with optimized combinations of salts and additives (e.g. Radstrom *et al.* 2008). *Taq* polymerases such as AmpliTaq Gold™ (Applied Byosistems), FastStart *Taq* DNA Polymerase (Roche), Platinum® *Taq* DNA polymerase (Invitrogen), TrueStart™ *Taq* DNA Polymerase (Fermentas), AccuSure™ DNA Polymerase (Bioline), Phusion™ High-Fidelity DNA Polymerase (Finnzymes) are a list of good examples (see Box 2).

DNA is present everywhere in a laboratory, especially where PCRs are frequently performed because amplified fragments persist as aerosols. Design of species-specific primers reduces the risk of amplification of nonspecific fragments and external DNA from human, prey items or bacteria (particularly in faecal material). Primers that do not amplify nontarget species (e.g. humans) can be designed. This is increasingly feasible thanks to increasing availability of sequence data from many species and software programs to align and compare multiple sequences.

Improved primer design with conventional software, such as Primer 3 and a number of later adaptations (Rozen & Skaletsky 2000; Kim & Lee 2007; Koressaar & Remm 2007), PERLPRIMER (Marshall 2007) or SNPBOX (Weckx *et al.* 2005) and highly specific multiplex primer design tools are now available on the web. The server Primerstation for the human genome (http://ps.cb.k.u-tokyo.ac.jp Yamada *et al.* 2006), the program MULTIPLX (Kaplinski *et al.* 2005) and the packages PRIMO (from BioToolKit 320; Chang Bioscience) and PrimerPremier (PREMIER Biosoft) are examples of effective ways for designing specific primers in large-scale analyses.

Precautions such as those in ancient DNA laboratories should be followed to prevent and monitor for contamination. Gilbert *et al.* (2005) describe nine criteria for working with ancient DNA and categorize risk factors associated with different projects. The criteria include isolation of work areas, use of negative controls for extractions and amplifications, amplification of only small segments, reproducibility, use of cloning of products to assess damage and contamination, independent replication, preservation of co-occurring biomolecules, quantification of DNA and evaluation of associated remains. They also consider hominid projects being the riskiest, followed by projects on cultivars and domestic animals, with low-risk projects involving projects on other wildlife species. Among the most important precautions, PCR set up should never be performed in the same day or just after conducting PCR or entering a room with PCR machines or post-PCR samples (see Fig. 1). Amplifying additional loci that work in possible contaminant species might also allow identifying contamination that remains undetected in the analysis of the target markers (e.g. Wandeler *et al.* 2003). For example, because of the high copy number of mitochondrial molecules, using mitochondrial specific primers in both samples and controls may be a sensitive way to monitor for contamination when working with nuclear DNA (Pusch *et al.* 1998).

Design of PCR protocols that minimize manipulation can reduce contamination risk. One could, for example, develop multilocus assays to successfully work using the

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Box 2. Polymerase enzymes for PCR

Presently, there are several hundred companies selling over 20 kinds of polymerase enzymes. There are two main characteristics that a polymerase enzyme must have that are crucial for amplifying small amounts of DNA: fidelity and 3′ → 5′ exonuclease activity (proofreading). Fidelity is particularly important when sequencing to detect SNP's. Heterozygous nucleotide sites must be unambiguously identified (in diploid individuals) or, for example, the false discovery rate of SNPs might be high.

Proofreading with 3′ → 5′ exonuclease activity is lacking in some polymerases [e.g. in *Thermus aquaticus* (*Taq*)] and sequencing error rates are higher than for polymerases with exonuclease activity [e.g. isolated from *Pyrococcus furiosus* (*Pfu*), *Thermococcus litoralis* (*Vent*), *Pyrococcus woesei* (*Pwo*)], which are often designated as high-fidelity polymerases. Studies comparing regular *Taq* polymerase vs. high-fidelity polymerases, such as the *Pfu*, report far lower error rates for the high-fidelity enzymes (Hansen *et al*. 2001).

Microsatellite genotyping with high-fidelity polymerases also gives lower error rates (Hite *et al*. 1996). When genotyping microsatellite loci (mostly dinucleotide), annoying stutter products are often formed during the PCR amplification. The primary cause of 'stutter' bands is a change in the number of repeat units because of slip-strand extension by *Taq* DNA polymerase. However, the use of high-fidelity polymerases (e.g. *Pfu*, *Vent*) reduces the formation of stutters as 3′ → 5′ exonuclease activity removes 3′ nontemplate nucleotides (Hite *et al*. 1996).

A study testing different polymerase enzymes (Spitaleri *et al*. 2004), showed that, for low template quantities, the regular *Taq* polymerases perform poorly and, for example, can increase allele dropout rates. However, in the same study, the engineered polymerases (e.g. AmpliTaq Gold) maintained high fidelity and sensitivity at very low DNA concentrations.

Amplification performance is another important characteristic. In this respect, it is well demonstrated that engineered DNA polymerases perform much better with low quality DNA. This is mainly because engineered DNA polymerases allow for the PCR hot-start technique. Hot start greatly increases the specificity and sensitivity of DNA amplification by avoiding competing side reactions during pre-PCR setup that can be initiated the moment that all reactants have been mixed and misprimming occurs.

At least two kinds of inactive polymerases are presently commercialized and often used in noninvasive studies: (i) recombinant DNA polymerase (e.g. AmpliTaq Gold® *Taq* DNA Polymerase; Roche Molecular Systems) engineered to be activated at temperatures higher than 90 °C, and (ii) Anti-*Taq* DNA polymerase antibodies, which inhibit polymerase activity at room temperature (e.g. Platinum® *Taq* DNA Polymerase; Invitrogen).

minimum number of single-tube reactions, as it would imply less manipulation for higher quantity of data produced per sample. RTQ-PCR has no post-PCR handling (e.g. gel electrophoresis) and so tubes are not opened after PCR, which minimizes DNA molecules in the laboratory (Nazarenko *et al*. 2002). RTQ-PCR also allows the real-time monitoring of target DNA amplification (Box 1) as well as direct scoring of the desired results (e.g. melt curve analysis, which can detect nontarget amplification).

Negative controls are essential to monitor contamination. Several blanks should be placed in the beginning (to monitor for environmental and/or reagents contaminations) and in the middle and end (to detect cross-contamination) of a series of samples (Borst *et al*. 2004). Minimizing PCR cycles (e.g. to 35 cycles) can reduce contamination risks because tiny amounts of contamination would unlikely lead to visible PCR products on electropherograms or gels. Human forensic laboratories typically limit their PCR cycles to <35. However, this can be problematic for degraded DNA samples, which can require 40–45 PCR cycles.

Mixed samples can cause errors in noninvasive genetics but can be detected and avoided using recent techniques and software (Roon *et al*. 2005). Great efforts are made to solve problems of DNA mixtures because more than one donor is frequently responsible for the material recovered from a forensic scene (e.g. in a rape, DNA from the victim and the aggressor might be collected simultaneously). In this context, novel computational programs have been developed to separate admixed genotypes, such as PENDULUM (Bill *et al*. 2005) or MAIES (Cowell *et al*. 2006) that are based on different models to analyse peak area values on electropherograms. DNA mixture should not be regarded as a major limitation, because, if >6–8 highly polymorphic microsatellites are genotyped, it is likely that some loci will have three alleles, which is impossible for diploid species, and thus would indicate possible contamination. Many wildlife and conservation based studies that identify mixed samples simply discard these samples in favour of those that indicate only one animal deposited the sample.

Post-PCR and genotyping errors

The most insidious problem in noninvasive genetics is genotyping errors. We define a genotyping error as a dif-

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ference between the true genotype and the inferred genotype (Pompanon *et al.* 2005; Luikart *et al.* 2008b), which does not include failed PCRs or failed DNA extractions. Amplification failure (no PCR product) is not as problematic as a genotyping error (erroneous genotype) because mistakes in data interpretation are less likely from failed PCRs. Genotyping error detection and avoidance (e.g. by using the multi-tubes approach) have been thoroughly reviewed elsewhere (Pompanon *et al.* 2005), and so below we summarize and update the available information, and highlight the main problems and ways to avoid them.

Three main kinds of genotyping errors are generally reported as: (i) allelic dropout (stochastic detection of false homozygotes at heterozygous loci because of failure of one allele to amplify), (ii) false alleles (creation of new alleles caused by slippage events of *Taq* polymerase during early cycles of PCR, that may reach a concentration similar to the authentic alleles when limited template exists), and (iii) Human error, the incorrect identification of alleles as a result of cross-contamination in the field or in the laboratory or database manipulation errors (Hoffman & Amos 2005; Pompanon *et al.* 2005). Human errors in data entry and manipulation (e.g. in spread sheets) are often the most frequent cause of genotyping errors (Paetkau 2003; Schwartz *et al.* 2006). Among the nonhuman-induced errors, allelic dropout is usually the most common error.

Extremely dissimilar error rates (depending on species, season of the year and sample type) have been documented, ranging from as low as 0–2% in faecal analysis (Bonin *et al.* 2004; Maudet *et al.* 2004) and 10% in human buccal samples (Whitaker *et al.* 2001) to approximately 24% in some carnivore faeces (Johnson & Haydon 2007), and over 30% in shed hairs (Gagneux *et al.* 1997). However, comparison of rates is challenging as some laboratories are more conservative in discarding samples, while others readily discard samples that show even the slightest sign of failure. These decisions dramatically change the reported error rate.

There are four main approaches used to handle genetic errors from noninvasive samples. The first and the most common is called the multiple tubes approach first developed by Navidi *et al.* (1992) and Taberlet *et al.* (1996), which suggests that 6–10 similar genotypes should be obtained for a locus to define an individual as homozygous or heterozygous (see also Miller *et al.* 2002). Here, each sample at each locus is run multiple times to ensure genotype consistency. Some form of this approach is used in almost every noninvasive study. However, while multi-tubing will detect genotyping errors, it can exhaust the DNA extracted and is fiscally expensive. In addition, the multi-tube approach may increase errors as samples are handled more often (inducing human error) and there are more chances to produce false alleles, which can be interpreted as a missing allele (allelic dropout). In addition, multi-tubing does nothing to prove that the existing database is error free.

A second approach is to quantify the amount of target, amplifiable nuclear DNA in the sample (Morin *et al.* 2001). Once this quantity is known, the appropriate number of multi-tube re-runs can be conducted. Morin *et al.* (2001) recommended that if a sample has <25 pg (of amplifiable DNA) per reaction, it should be discarded; if it has 26–100 pg per reaction, then seven repeat genotypings of the sample are necessary; if it has 101–200 pg per reaction then four repeats are required; and if >200 pg per reaction, only two repeats are necessary (see also Box 1).

A third approach has been to use computer algorithms to detect genotyping errors. Depending on the data and goal of the study, various algorithms have been suggested (Ewen *et al.* 2000; Miller *et al.* 2002; Valière 2002; Van Oosterhout *et al.* 2004; McKelvey & Schwartz 2005; Kalinowski 2006). Some of these examine deviations from Hardy–Weinberg proportions, others use pedigree information to catch errors, while others use the number of mismatches in recaptures (i.e. genotypes identified more than once and differing by only one or two alleles; McKelvey & Schwartz 2005) as an error signal. A recent paper suggests that sample-specific errors (only a few poor quality individual samples) can cause significant deviations from Hardy–Weinberg proportions; such samples should be identified and often discarded (Miquel *et al.* 2006). Some of the most widely used software tools for detecting and avoiding genotyping errors are provided in Table 2.

The fourth error handling approach is to model various error rates in the final statistical analysis. For example in capture–mark–recapture studies, Lukacs & Burnham (2005) derived a method to incorporate the probability of genotyping error into the closed-population models of Otis *et al.* (1978), Huggins (1989) and Pledger (2000) using the disproportionate number of genotypes collected once relative to genotypes collected more frequently to estimate error. These approaches have been developed for estimating animal abundance, but are relatively rare in population genetic studies. Another example is in parentage studies where accommodating genotyping errors during likelihood computations can improve paternity analyses, as has been shown using the software Cervus (Kalinowski *et al.* 2006). In a related study, Wang (2004) developed likelihood methods to infer full- and half-sibships from marker data with a high error rate and to identify typing errors at each locus in each reconstructed sib family.

It is important to note that blood and tissue samples are too often assumed to always yield low genotyping error rates. However, error rates can be substantial if

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Table 2 Some examples of the most widely used methods and software programs developed mainly for detecting and preventing genotyping errors

	Main functions					
Software	Identifying problematic samples	Estimating the number of multitube repeats	Identifying problematic loci allelic dropout, null alleles	Testing for HWE departures	Identifying mixed samples	References
Quality Indexes*	✔		✔			Miquel *et al.* (2006)
Gemini†	✔	✔	✔		✔	Valière *et al.* (2002)
Hw-QuickCheck				✔		Kalinowski (2006)
Pedmanager‡	✔		✔	✔		Ewen *et al.* (2000)
Cervus			✔	✔	✔	Marshall *et al.* (1998)
Gimlet	✔		✔			Valière (2002)
Reliotype	✔		✔		✔	Miller *et al.* (2002)
MICRO-CHECKER			✔	✔	✔	Van Oosterhout *et al.* (2004)
Dropout§	✔		✔			McKelvey & Schwartz (2005)
PENDULUM					✔	Bill *et al.* (2005)
Pedant		✔				Johnson & Haydon (2007)
GENECAP¶	✔		✔	✔	✔	Wilberg & Dreher (2004)

*Program available upon request from the authors.
†Simulation-based method to detect consensus genotypes.
‡When pedigree information is available.
§Bimodal test for loci that cause many samples to differ by only one allele.
¶This is just a Microsoft Excel macro.

these (normally high quality) samples are poorly preserved (Hoffman & Amos 2005). Comparative analysis of genotyping errors for noninvasive and assumed good quality DNA is helpful and needed (Soulsbury *et al.* 2007), but should be interpreted with caution.

Regarding this, Johnson & Haydon (2007) developed a maximum-likelihood-based method for estimating error rates from a single replication of a sample of genotypes. Simulations show it to be accurate and robust. It is implemented in a computer program, PENDANT, which estimates allelic dropout and false allele error rates with 95% confidence regions from microsatellite genotype data and performs power analysis. Finally, as mentioned in the previous section, mixed samples (with DNA from more than one individual) can be identified and computational programs have been developed to resolve genotypes, such as PENDULUM (Bill *et al.* 2005) or MAIES (Cowell *et al.* 2006).

Perspectives

The most promising areas for future research and development in noninvasive genetic studies involve large-scale PCR multiplexing techniques, massively parallel sequencing technologies, and more holistic studies including diet and parasite or disease analyses. Future multiplexing techniques should allow analysis of tens to hundreds of loci (Porreca *et al.* 2007; Meyer *et al.* 2008) on noninvasive samples (see also Box 3). This would vastly increase the statistical power of noninvasive approaches and facilitate use of massively parallel sequencing while making possible the targeted sequencing of interesting segments of the genome (e.g. exons under selection).

New SNP multiplex genotyping systems use tiny volumes (nanolitres) for SNP genotyping assays (e.g. TaqMan; ABI), which reduces the costs of reaction chemicals by nearly 98%, while automating and speeding up the genotyping process. For example, a new multiplex system using SNP chips from Fluidigm at BioMark™ (http://www.fluidigm.com/applications/ genotype-profiling.html) allows simultaneous genotyping of 48 or 96 SNP loci on each of 48 or 96 individuals at a cost of only US$0.10–0.20 per SNP (Perkel 2008). These systems, however, also require an initial investment in equipment often of the order of US$50 000–300 000.

Massively parallel sequencing technologies, e.g. 454 pyrosequencing by synthesis, and sequencing by ligation (Ellegren 2008; Shendure & Li 2008), should improve noninvasive studies because they work well on short DNA fragments typical of difficult and ancient DNA (Green *et al.* 2006). The main disadvantage of these sequencing technologies is that they do not allow easy sequencing of many individuals (samples), and the cost per sequencing run is thousands of dollars. However, costs are declining and clever study design can allow an entire study to be conducted on a single sequencing run, thereby minimizing total costs.

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Box 3. Multiplex PCR techniques

Multiplex PCR amplification has great untapped potential to improve noninvasive sampling by reducing cost, increasing speed and reducing consumption of DNA from typically low quantity sources (Henegariu *et al.* 1997; Butler 2005). Reducing manipulation and handling (fewer PCRs per individual sample) also minimizes the possibility of contamination and error during reaction setup.

Optimization of multiplex assays generally requires more time and effort than standard single plexes, because it involves designing primer pairs that do not interact and at the same time anneal under the same conditions. Optimization also sometimes requires, adjusting primer pair concentrations to give similar amounts of PCR product, choosing fluorescence labels for sets of loci according to their allele or size range, and combining all these aspects in an efficient and low-cost protocol (e.g. Guo & Milewicz 2007). Whenever possible, loci more difficult to amplify should be labelled with the highest energetic labels (e.g. blue fluoresces brighter than red). Once obtained, multiplexes greatly facilitate genotyping of large population samples rapidly and at reduced cost.

In forensics and noninvasive genetic studies, multiplex PCR is being used more for both microsatellites and SNPs (Morin & Mccarthy 2007). Rapid and economical multiplex assays also exist for monitoring the international trade of protected species; for example, a multiplex of several species-specific primers allows the distinction among shark species (Shivji *et al.* 2005; Magnussen *et al.* 2007). Multiplexes have also been designed to study natural animal populations, e.g. a multiplex of 14 microsatellites in one PCR was developed for racoon, *Procyon lotor*, Fike *et al.* (2007).

Three main issues can facilitate multiplex PCR on noninvasive samples: (i) Recently developed commercial kits can facilitate co-amplification of 5–10 loci or more (Luikart *et al.* 2008b). These kits include a new buffer that reduces competition among loci and improves primer annealing. Multiplex PCR can be >30% cheaper than standard singleplex (Mukherjee *et al.* 2007); (ii) The use of algorithms and software to design improved primer sets with no primer interactions (Kaderali *et al.* 2003; Vallone & Butler 2004); and (iii) The use of universal fluorescent tails on the 5′ end of primers to label PCR products (Oetting *et al.* 1995; Neilan *et al.* 1997). Fluorescent labelling of one primer in a pair is expensive, ranging between US$100 and 150 (Schuelke 2000).

To reduce costs, Oetting *et al.* (1995) developed a single reaction nested PCR that allows easy and consistent genotyping and more homogeneous PCR amplification among loci. For each locus, PCR includes three different primers: a reverse primer, a forward primer with a 5′ tail (e.g. M-13 sequence), and the universal M-13 primer with fluorescent-labelling. During the first PCR cycles, the forward primer with tail hybridizes with the target DNA fragments and is incorporated into the products, and then temperature is lowered (53 °C) to allow the universal tail to anneal and incorporate fluorescence to the subsequent PCR products. With this technique, one can synthesize and use one labelled forward primer (M-13) for each of several loci in a multiplex PCR (Missiaggia & Grattapaglia 2006). At the same time, PCR multiplex amplification will be facilitated as the same forward primer (M-13) can give more even amplification among loci and provide better results for low template DNA (Schuelke 2000). Laboratories studying many species can benefit a lot from using a common universal labelled tail or tails. A cost reduction of ∼40% can be achieved in the amplification of 10 microsatellites when compared with conventional methods (Missiaggia & Grattapaglia 2006).

Most studies use the M13 sequence as the universal tail, but any sequence with no complementarity to target genome could be used (Neilan *et al.* 1997). For multiplexing several loci where some of them have overlapping size ranges, one can optimize the PCR reaction using different fluorescent tails (Missiaggia & Grattapaglia 2006; Guo & Milewicz 2007).

Single nucleotide polymorphism multiplex assays can work well using low quantity DNA, for example, 50 pg (Onofri *et al.* 2006). Mini-STRs (up to 150 bp) have also been penta-plexed revealing detection limits of 12.5 pg for artificially degraded human DNA (Meissner *et al.* 2007). In noninvasive wildlife studies, multiplex PCR is not widely used. However, Mukherjee *et al.* (2007) developed a multiplex protocol to identify tiger species from faeces using three small mtDNA fragments. The multiplex had a significant decrease in the number of false negatives compared with conventional PCR (especially in old faeces).

Increasingly holistic noninvasive genetic studies are possible. They combine multiple kinds of information (e.g. on diet, parasite load, parasite population genetics, as well as host genetics and physiological status [stress and reproductive hormone secretions]) allowing more valuable studies addressing multiple questions or by providing more complete information on individuals allowing new questions to be addressed. Valentini *et al.* (2009) used 454 pyrosequencing on chloroplast DNA from faecal samples to determine the diet of bears (as

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well as birds, snails and grasshoppers). They showed that DNA-based faecal diet analysis using universal primers (e.g. DNA barcoding) and pyrosequencing can help determine what plant species are consumed by an individual. In the future, noninvasive collection of spatially referenced faeces from across a landscape could allow a comprehensive study of a species (e.g. bears) in an area by the enumeration of individuals, identification of gender, examination of diet, estimation of parasite load and parasite transmission patterns among individuals and geographic areas. This type of information could become crucial to the management of species and their habitat.

Concluding remarks

Application of noninvasive genetic approaches is exciting and promising. The power and role of noninvasive genetics in molecular ecology and conservation genetics will continually increase, thanks to the advancements in each step of a noninvasive study (Fig. 1) including new technologies (e.g. massively parallel sequencing) and advancements from different disciplines (e.g. human and livestock health, and forensics). Nonetheless, noninvasive genetic studies still usually require more funding and efforts in the laboratory, compared with traditional genetic studies with high-quality DNA, to ensure low genotyping error rates. Monitoring the efficacy and error rate associated with each of the multiple steps in a noninvasive study is crucial to ensure success.

Among the greatest needs for additional research is to directly compare the relative performance of new and improved methods (e.g. for sample storage, DNA extraction and amplification) in multiple independent laboratories, taxa and sample types. The lack of independent and quantitative comparisons of techniques makes it difficult to provide advice on which methods are best for a given species, sample type and sample conditions (but see Schwartz & Monfort 2008, p. 242). Some techniques might be species-specific and environment dependent, but more studies are needed to assess this issue.

Research questions, including those that could be addressed previously only using high-quality samples, can now be addressed using noninvasive genetics, thanks to lower error rates and our ability to analyse more loci and more samples. For example, in many natural populations, it is increasingly feasible to estimate relatedness, infer parentage and reconstruct pedigrees, all of which require many loci and low genotyping error rates. Genetic monitoring (Schwartz *et al.* 2007), defined as the quantification of temporal changes in DNA-based estimators (e.g. population abundance or effective size), is also becoming more feasible because more samples can be genotyped with more loci, thereby increasing statistical power to detect reduced variation, changes in population size and immigration. In addition, noninvasive genetics continually improves the ability of law enforcement to detect illegal trafficking of animals (e.g. Manel *et al.* 2002) by providing more representative samples across populations and increasing recovery of DNA from confiscated samples.

We are on the cusp of answering long-standing ecological and evolutionary questions in rare and elusive species, thanks to improved noninvasive sampling and new technologies for analysing short DNA fragments (Morin & Mccarthy 2007; Millar *et al.* 2008). This includes questions about the genetic basis of local adaptation that can be addressed by using genome-wide scans (Wiehe *et al.* 2006) and population genomic approaches (Luikart *et al.*, 2003) requiring genotyping of many loci, which is becoming feasible in noninvasive genetics. It also includes questions about how landscape features influence gene flow and dispersal in natural populations, which is a main goal of landscape genetics, an emerging approach that combines landscape ecology and population genetics (Manel *et al.* 2003). Landscape genetics typically requires analyses of hundreds of samples widely dispersed across landscapes; this is feasible only via noninvasive genetic approaches in some taxa.

In disease ecology, we will be able to estimate transmission rates and address questions about landscape features or environmental variables influencing disease spread, by noninvasively sampling of parasites (or parasite DNA) from hosts (Archie *et al.* 2009). For example, many microparasites (bacteria and viruses) and macroparasites (helminthes) are environmentally transmitted (shed into the environment) and can be obtained from faeces, urine or saliva. We can even conduct population genomic studies on parasites (e.g. to identify genes influencing transmission or virulence) for wildlife disease that are notoriously difficult to study because they require capture of many individuals, which is difficult or impossible, as described earlier for elusive, rare or dangerous wildlife species.

Overall, the recent boom in technological advances is rapidly advancing the relatively new field of noninvasive genetics. These new technologies are often derived from human-based fields such as medicine and genomics. The challenge for molecular ecologists will be keeping up with and integrating these rapidly changing fields and technologies to aid in the study and monitoring of wild populations.

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